IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mitani et al.	Examiner:	Bertagna, Angela
Serial No.:	10/532975	Group Art Unit:	1637
Filed:	April 28, 2005	Docket No.:	20078.0005USWO
Title:	PROCESS FOR AMPLIFYING NUCLEIC ACIDS		

DECLARATION UNDER 37 CFR §1.132

Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

I, Takefumi Ishidao, hereby declare as follows:

1. I graduated from
1996, March; Department of Biological Science, Faculty of Science, Kumamoto University.
1998, March; Master's Course, Graduate School of Science and Technology, Kumamoto University.
2001, March; Doctor's Course, Department of Biological Sciences, Graduate School of Science, and Faculty of Science Osaka University.

2. I have worked in
2001, April; Department of Molecular and Genetic Information, Institute of Molecular and Cellular Biosciences, Tokyo University
2004, April; Laboratory of Molecular Genetics, RIKEN Tsukuba Institute
2007, September; Kabushiki Kaisya DNAFORM.

3. I consider myself to be an expert in the field of Molecular biology.
Knockout mice, Cancer Research and Cell division.

4. Under my direction, the following comparative experiments were conducted for the purposes of demonstrating the effects of primers that satisfy the conditions of $10 \leq X \leq 30$,

$-1.00 \leq (X-Y)/X \leq 0.75$ and $30 \leq X+Y \leq 50$, as required by claims 1 and 9 in US Patent Application No. 10/532975. In particular, amplification studies of the sY160 gene using Human DNA as a template were conducted.

Experiment

1. Experimental Period

From January 14, 2009
To February 2, 2009

2. Experimental Site

RIKEN Yokohama Institute, West Research Building, Room No. W320
RIKEN Yokohama Institute, South Research Building, Room No. S106

3. Experimenter

Kabushiki Kaisha DNAFORM
Takefumi ISHIDAO

4. Experiment Objective

To demonstrate the effects of primers that satisfy the conditions of $10 \leq X \leq 30$, $-1.00 \leq (X-Y)/X \leq 0.75$ and $30 \leq X+Y \leq 50$, as required by claims 1 and 9 in US Patent Application No. 10/532975 by amplifying the sY160 gene using Human DNA (manufactured by Clontech) as a template.

5. Experimental Method

In this example, it was attempted to amplify the sY160 gene using Human DNA (manufactured by Clontech) as a template. The primer used was as described below. We asked Operon Biotechnologies to synthesize these primers.

The properties of the primers used for the experiments are described below. Furthermore, the relationships of respective primers to the template were as illustrated in FIG. 1. In this connection, underlined parts in the following sequences represent 3'-end regions common to each of sense primers and antisense primers, respectively.

Primer Set 1: a combination of a sense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (13mer) in the 5'-end side is hybridized with a region starting from 27 bases downstream of the 3'-end portion of the primer on a strand extended from the primer, and an antisense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (13mer) in the 5'-end side is

hybridized with a region starting from 21 bases downstream of the 3'-end portion of the primer on a strand extended from the primer:

SY160LP13 ATTCGATTCCGTTTACGGGTCTCGAATGGAATA

SY160RP13 CTAAATCGAATGGTCATTGCATTCCTTTCCATT

Primer Set 2: a combination of a sense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (13mer) in the 5'-end side is hybridized with a region starting from 6 bases downstream of the 3'-end portion of the primer on a strand extended from the primer, and an antisense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (13mer) in the 5'-end side is hybridized with a region starting from 10 bases downstream of the 3'-end portion of the primer on a strand extended from the primer:

SY160LP13-1 TTCCATTCCATATTACGGGTCTCGAATGGAATA

SY160RP13-1 GGAATGGAATTGATCATTGCATTCCTTTCCATT

Primer Set 3: a combination of a sense primer in which after annealing of a sequence (15mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (13mer) in the 5'-end side is hybridized with a region starting from 36 bases downstream of the 3'-end portion of the primer on a strand extended from the primer, and an antisense primer in which after annealing of a sequence (15mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (13mer) in the 5'-end side is hybridized with a region starting from 34 bases downstream of the 3'-end portion of the primer on a strand extended from the primer:

SY160LP13-2 TTCTATGACATTCGTCTCGAATGGAATA

SY160RP13-2 GAATAGAATCGAAGCATTCCTTTCCATT

Primer Set 4: a combination of a sense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (13mer) in the 5'-end side is hybridized with a region starting from 57 bases downstream of the 3'-end portion of the primer on a strand extended from the primer, and an antisense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (13mer) in the 5'-end side is hybridized with a region starting from 57 bases downstream of the 3'-end portion of the primer on a strand extended from the primer:

SY160LP13-3 ATGTTTTTGCATTCTACGGTCTCGAATGGAATA

SY160RP13-3 TCGAATGGAATGGTCATTGCATTCCTTTCCATT

Primer Set 5: a combination of a sense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (16mer) in the 5'-end side is hybridized with a region starting from 27 bases downstream of the 3'-end portion of the primer on a strand extended from the primer, and an antisense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (16mer) in the 5'-end side is hybridized with a region starting from 21 bases downstream of the 3'-end portion of the primer on a strand extended from the primer:

SY160LP16 GACATTCGATTCCGTTTACGGGTCTCGAATGGAATA

SY160RP16 GAACTAAATCGAATGGTCATTGCATTCCTTTCCATT

Primer Set 6: a combination of a sense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (16mer) in the 5'-end side is hybridized with a region starting from 34 bases downstream of the 3'-end portion of the primer on a strand extended from the primer, and an antisense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (16mer) in the 5'-end side is hybridized with a region starting from 10 bases downstream of the 3'-end portion of the primer on a strand extended from the primer:

SY160LP16-1 ATTCTATGACATTCGATACGGGTCTCGAATGGAATA

SY160RP16-1 AATGGAATGGAATTGATCATTGCATTCCTTTCCATT

Primer Set 7: a combination of a sense primer in which after annealing of a sequence (15mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (16mer) in the 5'-end side is hybridized with a region starting from 36 bases downstream of the 3'-end portion of the primer on a strand extended from the primer, and an antisense primer in which after annealing of a sequence (15mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (16mer) in the 5'-end side is hybridized with a region starting from 34 bases downstream of the 3'-end portion of the primer on a strand extended from the primer:

SY160LP16-2 ACATTCTATGACATTCGTCTCGAATGGAATA

SY160RP16-2 AGTGAATAGAATCGAAGCATTCCTTTCCATT

Primer Set 8: a combination of a sense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (16mer) in the 5'-end side is hybridized with a region starting from 52 bases downstream of the 3'-end portion of the primer on a strand extended from the primer, and an antisense primer in which after annealing of a sequence (20mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (16mer) in the 5'-end side is

hybridized with a region starting from 46 bases downstream of the 3'-end portion of the primer on a strand extended from the primer:
SY160LP16-3 TTTTTTGCATTCCATTCTACGGTCTCGAATGGAATA
SY160RP16-3 GAATGGAATGGAAGTGTCATTGCATTCCTTTCCATT

Primer Set 9: a combination of a sense primer in which after annealing of a sequence (16mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (13mer) in the 5'-end side is hybridized with a region starting from 33 bases downstream of the 3'-end portion of the primer on a strand extended from the primer, and an antisense primer in which after annealing of a sequence (16mer) placed in the 3'-end side of a primer to the template and extension reaction, a sequence (13mer) in the 5'-end side is hybridized with a region starting from 33 bases downstream of the 3'-end portion of the primer on a strand extended from the primer:
SY160TP-F(16,32) TATGACATTCGATGGTCTCGAATGGAATA
SY160TP-R(16,32) TGAATAGAATCGACATTCCTTTCCATTCC

Tables 1, 2, and 3 are shown below in which properties of the above-mentioned primer sets 1 to 9 are summarized. In the following tables 1 to 3, "○" denotes that the primer satisfies the following mathematical formula 1 or 2 described in the corrected claims of JP 3867926 (hereinafter referred to as a "present patent"). Furthermore, "×" denotes that the primer does not satisfy the following mathematical formula 1 or 2. In this case, as can be seen from the following tables 1 to 3, all the primers satisfy the condition of $10 \leq X \leq 30$.

Mathematical Formula 1: $-1.00 \leq (X-Y)/X \leq 0.75$
Mathematical Formula 2: $30 \leq X+Y \leq 50$

[Table 1]

Primer Set	Primer	Formula	X and Y	(X-Y)/X	X+Y
1	SY160LP13	1 : o 2 : o	X=20, Y=26	-0.3	46
	SY160RP13		X=20, Y=20	0	40
2	SY160LP13-1	1 : o 2 : ×	X=20, Y=5	0.75	25
	SY160RP13-1		X=20, Y=9	0.55	29
3	SY160LP13-2	1 : × 2 : o	X=15, Y=35	-1.333	50
	SY160RP13-2		X=15, Y=35	-1.333	50
4	SY160LP13-3	1 : × 2 : ×	X=20, Y=56	-1.8	76
	SY160RP13-3		X=20, Y=56	-1.8	76

[Table 2]

Primer Set	Primer	Formula	X and Y	(X-Y)/X	X+Y
5	SY160LP16	1 : o 2 : o	X=20, Y=26	-0.3	46
	SY160RP16		X=20, Y=20	0	40
6	SY160LP16-1	1 : o 2 : ×	X=20, Y=33	-0.65	53
	SY160RP16-1		X=20, Y=9	0.55	29
7	SY160LP16-2	1 : × 2 : o	X=15, Y=35	-1.333	50
	SY160RP16-2		X=15, Y=33	-1.2	48
8	SY160LP16-3	1 : × 2 : ×	X=20, Y=51	-1.55	71
	SY160RP16-3		X=20, Y=45	-1.25	65

[Table 3]

Primer Set	Primer	Formula	X and Y	(X-Y)/X	X+Y
9	SY160 TP-F(16,32)	1 : o 2 : o	X=16, Y=32	-1	48
	SY160 TP-R(16,32)		X=16, Y=32	-1	48

<Amplification Experiment>

A reaction solution (25 μL) with the following composition was prepared: Tris-HCl (20 mM, pH 8.8), KCl (10 mM), $(NH_4)_2SO_4$ (10 mM), $MgSO_4$ (2 mM), Triton X-100 (0.1%), dNTP (0.4 mM), 100 pmol of each of the above-mentioned primer pairs, 100 ng of template DNA, and 8 U of Bst DNA polymerase (NEW ENGLAND BioLabs). This was incubated at 60°C for 90 minutes, 120 minutes, or 150 minutes.

Then 1 μl of each reaction solution was electrophoresed with 3% NuSieve GTG Agarose (manufactured by BMA; purchased from TAKARA BIO INC.; "NuSieve" is a registered trademark of BMA).

<Amplification Product Verification Test>

Subsequently to the above-mentioned amplification experiment, further an amplification product verification test was conducted as follows. That is, among the amplification products obtained in the above-mentioned amplification experiment, the amplification product that seemed to have highest amplification efficiency as an amplification

experiment object in each of the above tables 1, 2, and 3 was used and digested with a restriction enzyme. Conditions for digestion with the restriction enzyme were 37°C for 3 hours, and 1 μL of reaction solution of the amplification product obtained using each of the primer sets was digested with a restriction enzyme BstXI.

Each digested product was electrophoresed with 3% NuSieve GTG Agarose (manufactured by BMA; purchased from TAKARA BIO INC.; "NuSieve" is a registered trademark of BMA).

6.Experimental Result

<Description of Drawings>

Attached FIG. 1 shows the relationships of respective primers to the template in the primer sets 1 to 9.

Attached FIGS. 2 and 3 are electrophoregrams showing the results of the amplification experiments with the primer sets 1 to 8. The electrophoregram in FIG. 2 shows the results obtained with the primer sets 1 to 4, and the electrophoregram in FIG. 3 shows the results obtained with the primer sets 5 to 8. In each drawing, the numerical values indicated on the left side of the electrophoregram denote the sizes of the amplification products. Furthermore, in each drawing, the table shown below the electrophoregram indicates reaction conditions of each primer in the amplification experiment. In the table, with respect to the template, "present" indicates that the template was added as described above, while "absent" indicates that the same reaction was performed with no template added.

FIGS. 4 and 5 are electrophoregrams showing the results of the amplification product verification tests with the primer sets 1 to 8. The sample of each lane in each drawing is as shown in the table located on the right side of each drawing. The numerical values shown on the left side of the electrophoregram in each drawing indicate the sizes of each restriction enzyme digestion fragment estimated from each base sequence.

Attached FIG. 6 is an electrophoregram showing the result with respect to the primer set 9. The sample of each lane shown in FIG. 6 is as in the table indicated on the right side of FIG. 6. That is, lane 1 is a size marker. Lane 2 is an amplification product obtained when the reaction time was 90 minutes. Lane 3 is a sample obtained with the amplification product being treated with a restriction enzyme BstXI. Lane 4 is a sample obtained through reaction with no template added thereto.

<Amplification Experiment Results>

In the reaction with no template being added, no band other than that in which an unreacted primer was dyed was observed. The results thereof are shown in lanes 6, 11, 16, and 22 (primer sets 1 to 4) in FIG. 2, lanes 6, 11, 16, and 22 (primer sets 5 to 8) in FIG. 3, and lane 4 (primer set 9) in FIG. 6.

When the reaction time was zero minute although a template was added, no amplification product was observed. The results thereof are shown in lanes 2, 7, 12, and 18 (primer sets 1 to 4) in FIG. 2 and lanes 2, 7, 12, and 18 (primer sets 5 to 8) in FIG. 3.

In each of the primer sets 1, 5, and 9, which is composed of primers that satisfy both the mathematical formulae 1 and 2, a target amplification product was obtained sufficiently through a reaction in a short reaction time of 90 minutes after a template was added. The results thereof are shown in lanes 3, 4, and 5 (primer set 1) in FIG. 2, lanes 3, 4, and 5 (primer set 5) in FIG. 3, and lane 2 (primer set 9) in FIG. 6. Among small size bands, the band around 260 bp indicates a product anticipated by the synthesis reaction of the present invention.

In the primer set 2 composed of primers that satisfy the mathematical formula 1 but do not satisfy the mathematical formula 2, amplification products were obtained in the samples subjected to a reaction time of at least 90 minutes. Similarly, in the primer set 6 composed of primers that satisfy the mathematical formula 1 but do not satisfy the mathematical formula 2, an amplification product was obtained only in the sample subjected to a reaction time of 150 minutes. The results thereof are shown in lanes 8, 9, and 10 (primer set 2) in FIG. 2 and lanes 8, 9, and 10 (primer set 6) in FIG. 3. In this case, as described later, these amplification products are nonspecific amplification products.

In the primer set 3 composed of primers that satisfy only the mathematical formula 2 and do not satisfy the mathematical formula 1, an amplification product was obtained only in the sample subjected to a reaction time of 150 minutes. Similarly, in the primer set 7 composed of primers that satisfy only the mathematical formula 2 and do not satisfy the mathematical formula 1, no amplification product was obtained even in the sample subjected to a reaction time of 150 minutes. These results are shown in lanes 13, 14, and 15 (primer set 3) in FIG. 2 and lanes 13, 14, and 15 (primer set 7) in FIG. 3.

In the primer set 4 composed of primers that satisfy neither the mathematical formula 1 nor the mathematical formula 2, no amplification product was obtained even in the sample subjected to a reaction time of 150 minutes. Similarly, in the primer set 8 composed of primers that satisfy neither the mathematical formula 1 nor the mathematical formula 2, an amplification product was obtained only in the sample subjected to a reaction time of 150 minutes. These results are shown in lanes 19, 20, and 21 (primer set 4) in FIG. 2 and lanes 19, 20, and 21 (primer set 8) in FIG. 3.

<Amplification Product Verification Test Results>

In the primer sets 1, 5, and 9, each of which satisfies both the mathematical formulae 1 and 2, most of the bands in the undigested state were changed into those with sizes estimated to be obtained after digestion with the restriction enzyme. The results thereof are shown in lane 2 (primer set 1) in FIG. 4, lane 2 (primer set 5) in FIG. 5, and lane 3 (primer set 9) in FIG. 6. Thus, it was proved that target amplification products were obtained efficiently through reactions in a short reaction time of 90 minutes using those primer sets.

On the other hand, with respect to the amplification products obtained with the primer sets 2 and 6 that do not satisfy the mathematical formula 2, since the bands were not changed in size even after digestion, it was proved that they were nonspecific amplification products. The results thereof are shown in lane 3 (primer set 2) in FIG. 4 and lane 3 (primer set 6) in FIG. 5.

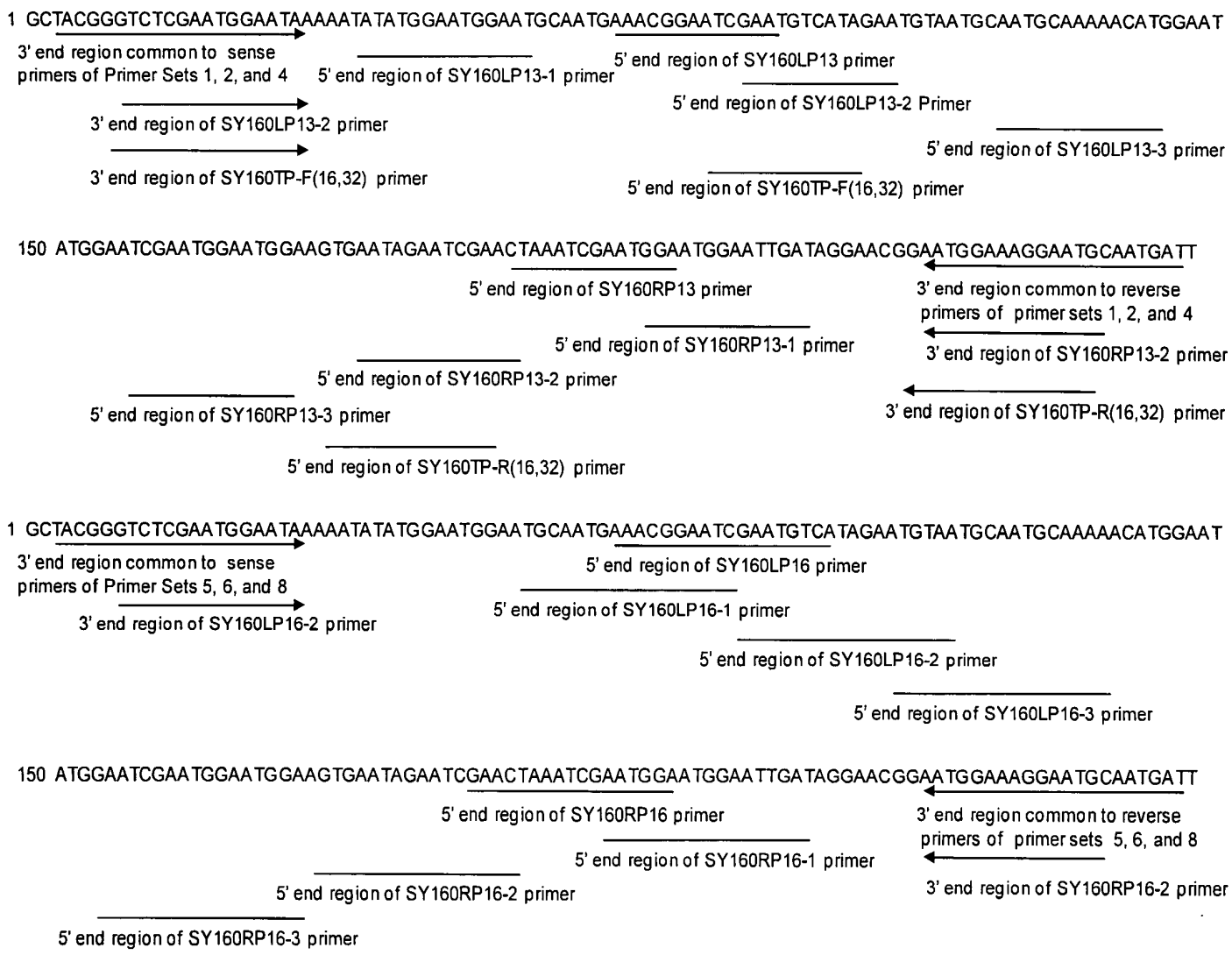
As described above, with the primer sets in which primers that satisfied both the mathematical formulae 1 and 2 were used, target amplification products were obtained efficiently through reactions in a short reaction time of 90 minutes. On the other hand, with the primer sets in which only primers that did not satisfy one or both of the mathematical formulae 1 and 2 were used, the amplification efficiency was lower or nonspecific amplification occurred. Thus, according to the inventions of the corrected claims of the present patent, it was proved that desired effects (performances) were obtained in the amplification of the sY160 gene using Human DNA as a template.

I declare under the penalty of perjury of the laws of the United States of America that the foregoing is true and correct to the best of my information and belief.

Signed this 21st day of May, 2009, at Yokohama, JAPAN

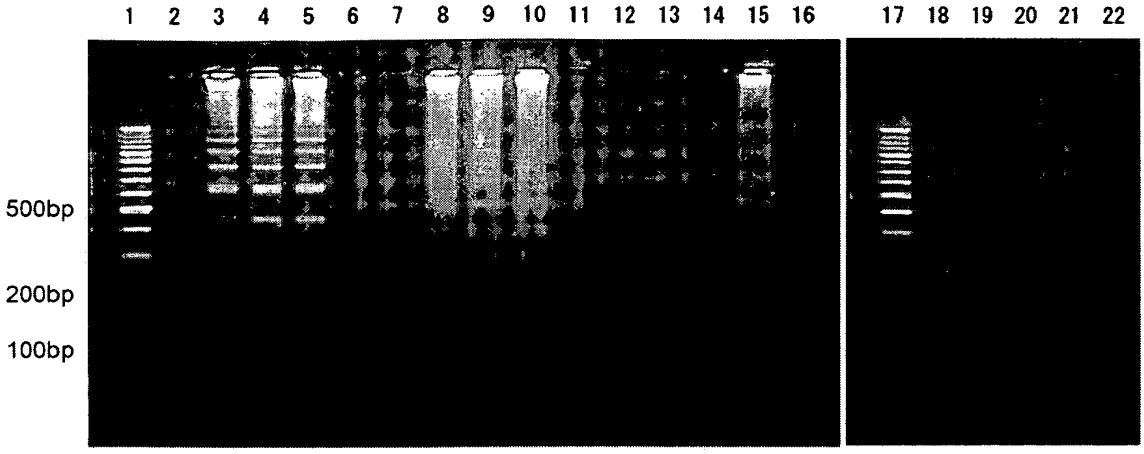
Takefumi Ishidao.

Takefumi ISHIDAO


1 GCTACGGGTCTCGAATGGAATAAAAATATATGGAATGGAATGCAATGAAACGGAATCGAATGTCATAGAATGTAATGCAATGCAAAAACATGGAAT
3' end region common to sense primers of Primer Sets 1, 2, and 4
5' end region of SY160LP13-1 primer
5' end region of SY160LP13 primer
5' end region of SY160LP13-2 Primer
3' end region of SY160LP13-2 primer
5' end region of SY160LP13-3 primer
3' end region of SY160TP-F(16,32) primer
5' end region of SY160TP-F(16,32) primer
150 ATGGAATCGAATGGAATGGAAGTGAATAGAATCGAACTAAATCGAATGGAATGGAATTGATAGGAACGGAATGGAAAGGAATGCAATGATT
5' end region of SY160RP13 primer
3' end region common to reverse primers of primer sets 1, 2, and 4
5' end region of SY160RP13-1 primer
3' end region of SY160RP13-2 primer
5' end region of SY160RP13-2 primer
5' end region of SY160RP13-3 primer
3' end region of SY160TP-R(16,32) primer
5' end region of SY160TP-R(16,32) primer
1 GCTACGGGTCTCGAATGGAATAAAAATATATGGAATGGAATGCAATGAAACGGAATCGAATGTCATAGAATGTAATGCAATGCAAAAACATGGAAT
3' end region common to sense primers of Primer Sets 5, 6, and 8
5' end region of SY160LP16 primer
5' end region of SY160LP16-1 primer
3' end region of SY160LP16-2 primer
5' end region of SY160LP16-2 primer
5' end region of SY160LP16-3 primer
150 ATGGAATCGAATGGAATGGAAGTGAATAGAATCGAACTAAATCGAATGGAATGGAATTGATAGGAACGGAATGGAAAGGAATGCAATGATT
5' end region of SY160RP16 primer
3' end region common to reverse primers of primer sets 5, 6, and 8
5' end region of SY160RP16-1 primer
3' end region of SY160RP16-2 primer
5' end region of SY160RP16-2 primer
5' end region of SY160RP16-3 primer

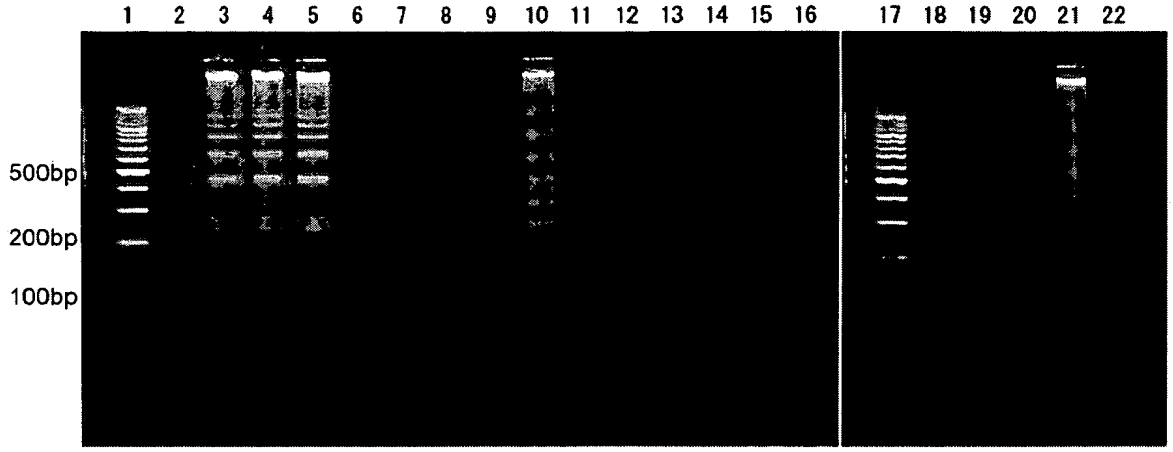
[FIG. 1]

[FIG. 2]



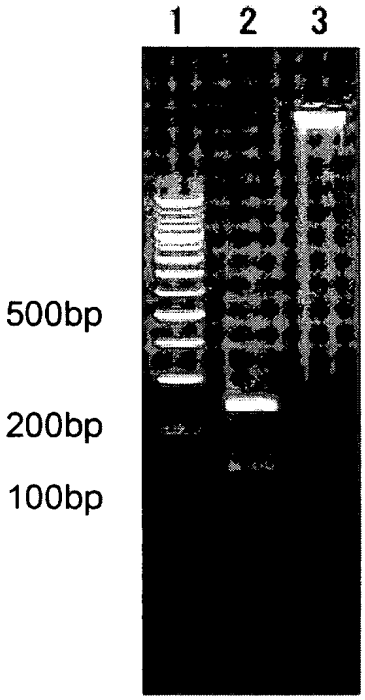
Lane	Primer	Template	Reaction Time (min)
1	Size Marker		
2	Primer Set 1	Present	0
3	Primer Set 1	Present	90
4	Primer Set 1	Present	120
5	Primer Set 1	Present	150
6	Primer Set 1	Absent	150
7	Primer Set 2	Present	0
8	Primer Set 2	Present	90
9	Primer Set 2	Present	120
10	Primer Set 2	Present	150
11	Primer Set 2	Absent	150
12	Primer Set 3	Present	0
13	Primer Set 3	Present	90
14	Primer Set 3	Present	120
15	Primer Set 3	Present	150
16	Primer Set 3	Absent	150
17	Size Marker		
18	Primer Set 4	Present	0
19	Primer Set 4	Present	90
20	Primer Set 4	Present	120
21	Primer Set 4	Present	150
22	Primer Set 4	Absent	150

[FIG. 3]



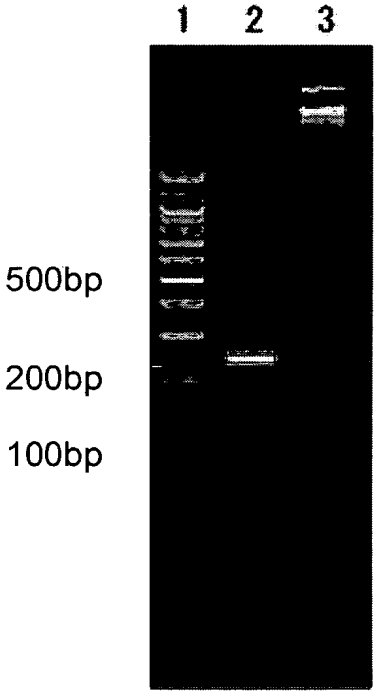
Lane	Primer	Template	Reaction Time (min)
1	Size Marker		
2	Primer Set 5	Present	0
3	Primer Set 5	Present	90
4	Primer Set 5	Present	120
5	Primer Set 5	Present	150
6	Primer Set 5	Absent	150
7	Primer Set 6	Present	0
8	Primer Set 6	Present	90
9	Primer Set 6	Present	120
10	Primer Set 6	Present	150
11	Primer Set 6	Absent	150
12	Primer Set 7	Present	0
13	Primer Set 7	Present	90
14	Primer Set 7	Present	120
15	Primer Set 7	Present	150
16	Primer Set 7	Absent	150
17	Size Marker		
18	Primer Set 8	Present	0
19	Primer Set 8	Present	90
20	Primer Set 8	Present	120
21	Primer Set 8	Present	150
22	Primer Set 8	Absent	150

[FIG. 4]



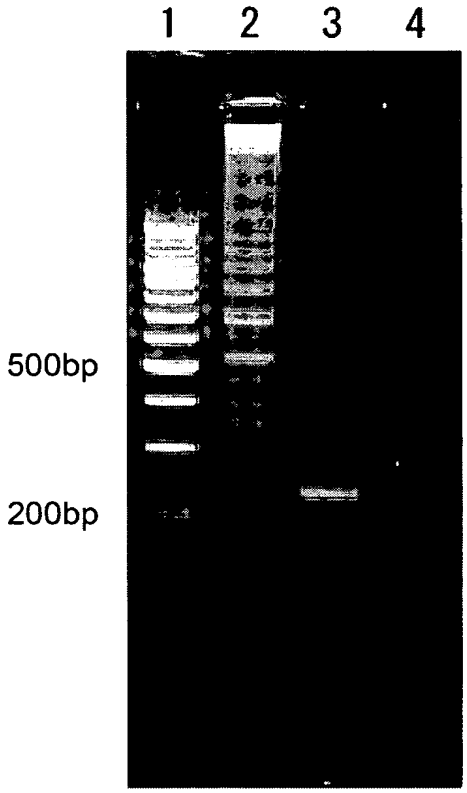
Lane	Primer Set	Reaction Time (min)
1	Size Marker	
2	Primer Set 1	90
3	Primer Set 2	90

[FIG. 5]



Lane	Primer Set	Reaction Time (min)
1	Size Marker	
2	Primer Set 5	90
3	Primer set 6	150

[FIG. 6]



Lane	
1	Size Marker
2	Amplification Product (90 min Reaction)
3	Restriction Enzyme BstXI Treatment
4	No Template